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(56) Documents Cited
WO 93/24626 A1 WO 93/08292 A1 WO 93/05815 A1
WO 91/07487 A1 WO 91/00359 A1
Bio/Technology 1992,10,286-291

(58) Field of Search
UK CL (Edition M) C3H HB7T HB7X
INT CL[6] A01K 67/027 , C12N 5/10 15/87
ONLINE DATABASES: WPI,CLAIMS,DIALOG/BIOTECH

(54) Introducing DNA into the germ line of birds

(57) Avian cells are transformed by introducing nucleic acid by particle bombardment ("biollistics"), e.g. using gold or tungsten particles of diameter 0.1 - 1.0 µm with adsorbed DNA. Preferably germinal crescent cells of an egg are transformed, normal development and hatching then giving a transgenic bird and transgenic progeny.

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At least one drawing originally filed was informal and the print reproduced here is taken from a later filed formal copy.

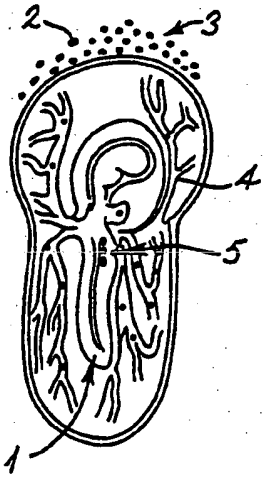


Fig. 1

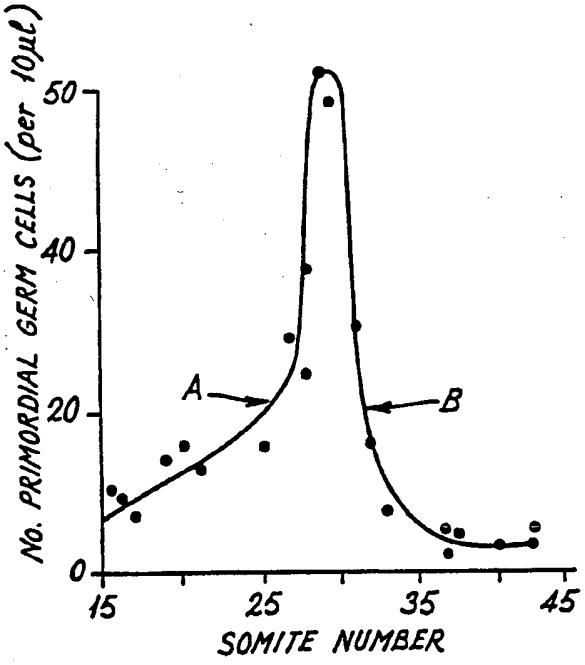


Fig. 2

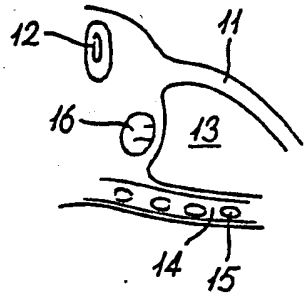


Fig. 3

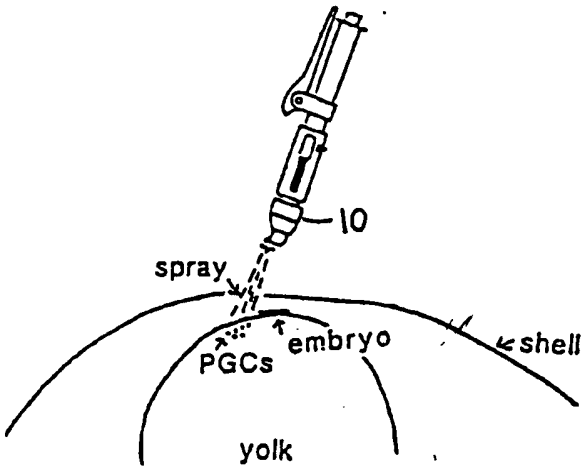


Fig 4A

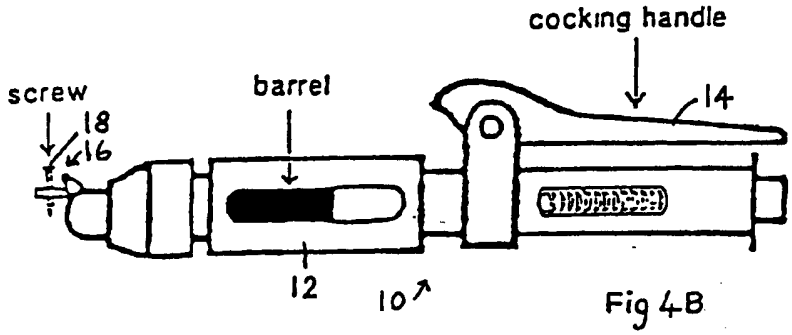


Fig 4B

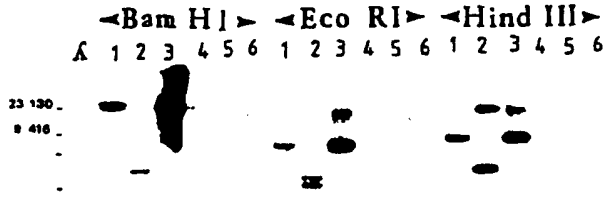


Fig 5

Fig 6

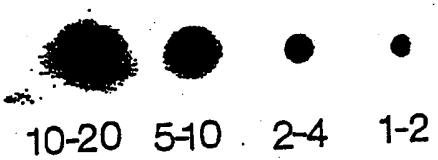
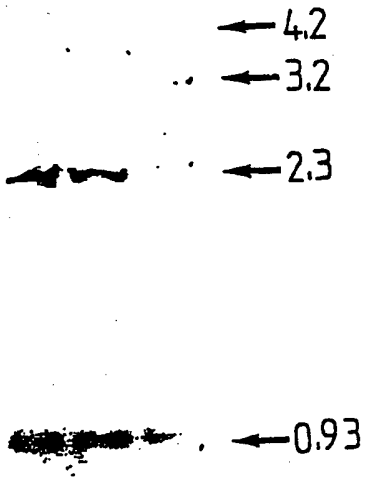
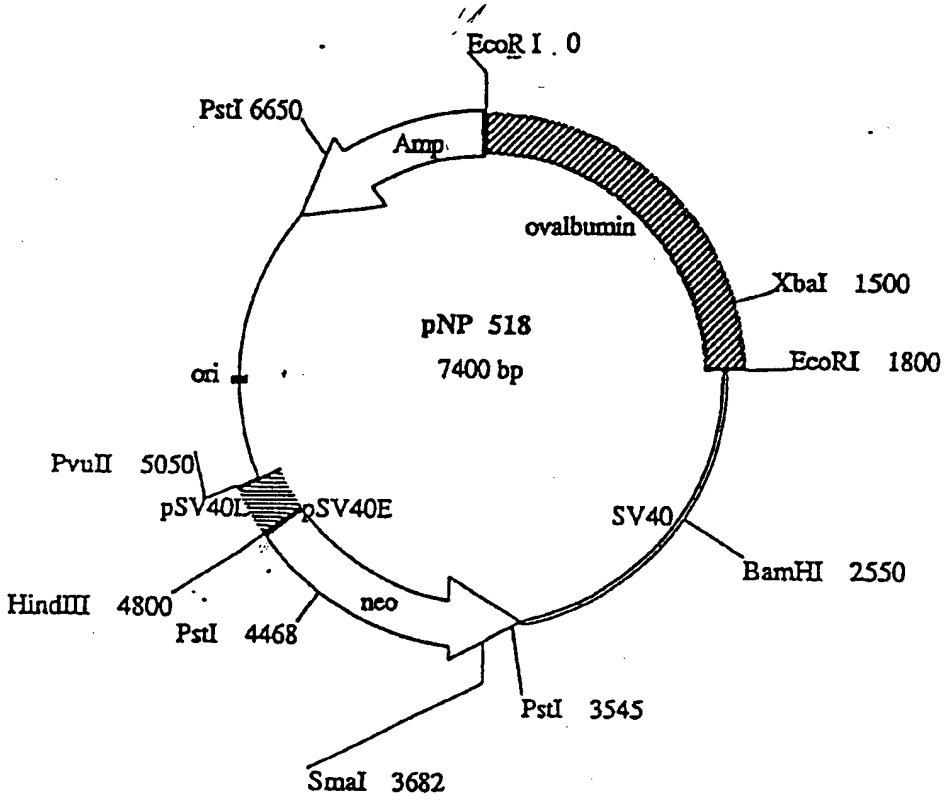


Fig 7

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HindIII 4800
PstI 4468
neo
PstI 3545
SmaI 3682

Fig 8

INTRODUCING DNA INTO THE GERM LINE OF BIRDS

This invention is in the field of transgenic birds.

There are several known ways to introduce exogenous (foreign) genes into embryos. These are by direct injection into the pronucleus, by infection of early embryos with genetically manipulated viruses or by the transfer of cells with a modified genotype into the blastocyst to produce a chimera. The value of each of these approaches varies with the nature of the embryological system involved and in this context birds have been particularly difficult to use.

Injection of genes into pronuclei has been successful in mice, rabbits, sheep etc. but attempts to produce transgenic birds by direct injection of DNA into the pronucleus have been largely unsuccessful because it is difficult to locate the pronucleus within the body of a large yolky egg, H. Sang & M.M. Perry. Molecular Reproduction & Development 1, 98-106 (1989). Infection of eggs with retroviruses is not a reliable method. Thus, it has been reported by R.A. Bosselman et al (of Amgen Inc.) Science 243, 533-535 (27 January 1989) that only 8% infection of the germ cell line occurred.

It has been found that foreign nucleic acid, especially foreign gene(s), can be introduced into

explanted primordial germ cells derived from the embryo of a donor bird, that the cells carrying the foreign nucleic acid can be introduced into the embryo of a recipient bird, and that the foreign nucleic acid is thereby carried into the germ cells of the embryo and therefore into the germ line of a bird which will be produced from the embryo. In this way, transgenic birds, especially poultry and game birds, can be produced. This is described in European Patent Application 90904874.6.

A relatively young technique for gene introduction into cells is the use of particle bombardment, or "biollistics", reviewed in Klein et al, Biotechnology, 10, 286-291 (1992). The technique was developed originally for the transfer of genes to plant cells, but has been used successfully to transfer genes to animal cells, in culture, in explanted tissue and in vivo. Only somatic cells have been targeted to date.

The present invention is founded on the surprising discovery that ballistic microprojectiles can be used successfully in the transfer of genes specifically to the germ line of birds. We are not aware of any previous suggestion that the approach might be useful for this purpose. Additionally, it was not obvious that it would work specifically in this context, for reasons which are discussed below.

According to the present invention there is a

method of introducing exogenous nucleic acid (e.g. DNA into the germ line of a bird, which comprises firing into the germinal crescent of a bird embryo particles which have the nucleic acid adsorbed on their surface.

Nucleic acids transferred in accordance with the invention can be RNA or DNA; DNA can be genomic or cDNA produced from genomic or mRNA. The nucleic acid can act as a genetic marker, it can promote recombination of introduced nucleic acid with cellular (e.g. genomic) nucleic acid or it can code for a protein. In all cases, appropriate promoters, signal and transcription-termination sequences may be required, as is recognized in the art. If the DNA is to be expressed, producing an encoded protein, control sequences can be included as appropriate. The nucleic acids can be gene sequences selected from those familiar to those skilled in the poultry genetics field, including growth genes, genes which may impart resistance to poultry diseases such as coccidiosis, Marek's Disease Virus, Newcastle Disease Virus, Infectious Bronchitis Virus, Infectious Bursal Disease Virus and so forth. Further, genes coding for attenuated strains of Salmonella may be of value.

The egg containing the embryo is to be open to allow access to the germinal crescents. Preferably, the opened shell is manipulated so that the embryo lies protected beneath the shell while the germinal crescents are exposed. Preferably the embryo is about two days old, so that the germinal crescent, containing primordial germ cells, can be exposed, lying outside the embryo. Some of the DNA introduced enters the primordial germ cells.

Following the firing of the particles into the germinal crescents the egg containing the embryo should be resealed and incubated until hatching of a live bird.

The particles must be small enough to be able to enter the primordial germ cells, dense enough to have sufficient mass to penetrate into the cells and of material which adsorbs DNA onto the surface. Typically metal such as tungsten or gold is suitable. Preferably the particles have a diameter of 0.1 to 1.0μm.

In a preferred embodiment of the present invention, a range of particles of different sizes, and therefore masses, is fired into the germinal crescent. This will ensure that some of the particles are of the

correct size to enter the cells and have the correct momentum imparted to them to stop in the cells. Some particles may go past the target cells, but in bird embryos this is unimportant because behind the germinal crescent there is only yolk, which will not be damaged if particles reach it.

Preferably, the particles are fired from a device which does not expose the tissue to vacuum, either partial or complete. While a device which fires particles through a vacuum enables prediction of impact effects because of greater uniformity in projection, damage to the tissue may be increased by the vacuum itself.

The DNA may include any sequence of interest, which may code for a protein or may simply act as a genetic marker. If the DNA is to be expressed, producing an encoded protein, control sequences may be needed, as appropriate. The DNA may include sequences which promote recombination of the introduced DNA with cellular, eg genomic, DNA.

The present invention can generally be performed several times a minute, allowing large scale transfer of genetic material to many bird embryos and subsequent growth into transgenic birds. This contrasts with known techniques which take time of the order of hours and involve extensive screening.

The invention includes all conventional further steps downstream of the introduction of the foreign nucleic acid to the recipient embryo, such as hatching chicks, rearing birds from these chicks, using the eggs of reared egg-laying birds as a source of genes for a further gene transfer and so on. The term "chick" herein denotes the newly hatched offspring of any bird not necessarily of a fowl unless the context so requires.

The invention further includes "indirect products of the method" viz. birds which are made transgenic or carry foreign nucleic acid in their germ line through application of the process of the invention to their mother or to any maternal ancestor of the bird's mother or father.

The invention is of significance chiefly for gene transfer using cells of the same species or even strain. It is preferably used in poultry such as chickens, ducks, turkeys, geese and guinea fowl or game birds such as pheasants, grouse and partridges.

The following Examples illustrate the invention with reference to the accompanying drawings. All stages of embryonic development are on the standard Hamburger - Hamilton scale.

Brief description of the drawings

Figure 1 shows a chicken embryo in surface view;

Figure 2 is a graph of concentration of primordial germ cells in blood plotted against time (stage of

embryonic development):

Figure 3 is a transverse section or part of a chick embryo showing the germinal ridge;

Figure 4 shows details of a modified Panjet (Fig 4A) and its use as a ballistic system for introducing plasmid DNA into the 2d embryo germ cells (PGC) showing the germinal ridge (Fig 4B);

Figure 5 shows southern blots identifying vector DNA in the sperm of cockerels grown from treated eggs (no's 1, 2 and 3) and control birds (no's 4, 5 and 6);

Figure 6 shows dot blots that demonstrate that the offspring resulting from the breeding of a bird ballistically treated as an embryo contains foreign DNA;

Figure 7 shows Southern blots of DNA from offspring positive for foreign DNA; and

Figure 8 shows a map of the plasmid used in ballistic treatment.

Primordial germ cells (PGCs) are the cells which are destined to give rise to gametes (eggs and Spermatozoa). They are large cells (larger than blood cells, for example), typically of diameter up to 20 micrometres. After an egg has incubated for about 4 hours, PGC's can be seen in the germinal crescent which lies outside the embryo (Figure 1). In Figure 1. the

donor embryo 1 has primordial germ cells 2 in the germinal crescent 3. The germinal crescent lies between the area opaca and area pellucida interior to the head of the developing embryo. Also shown are blood vessels 4 and the gonad 5. The PGCs migrate from this site of origin, via the bloodstream, to the site of the future gonad, which is called the germinal ridge. In the embryo of the domestic fowl, this migration occurs after about 50h of incubation at stage 16 of development (Figure 2: "A"). It is seen as a large pulse of transient primordial germ cells among the normal erythrocytes of the blood. Within a few hours they disappear from the blood and settle in the germinal ridge (Figure 2: "B"). Figure 2 shows the population of PGCs in the blood stream of the embryo plotted against somite number, which represents a stage of embryonic development. (The somite is a block of muscle. By counting these somites in the head to tail direction of the embryo, its development can be quantised). Figure 3 is a transverse section of a part of the embryo showing the germinal ridge. 1 = mesoderm, 12 = spinal cord, 13 = extra-embryonic cavity, 14 = blood vessel, 15 = PGCs carried in the bloodstream, 16 = germinal ridge.

Once settled in the germinal ridge, the PGCs proliferate to form germ cells. The number settling is of the order of a few hundred, while the number of proliferated germ cells produces is of the order of a million in the female. Only a fraction of these germ cells (a few thousand) are carried through to the adult bird, there being extensive atrophy of these oocytes after hatching.

Example

A needle-less injection syringe (10) shown in Fig 4 is a modification of a commercial article (Panjet, Dundee) that is designed to deliver anaesthetics to human tissues. It has been modified by reducing the strength of the spring, decreasing the barrel volume and correcting the dispersal of the spray. In the modified form it will delivery 10-20μl through the overlying albumen (c 1-2mm) into the 2 day (2d) chick embryo. The syringe 10 has a barrel 12, a plunger mechanism operated via a cocking handle 14, and a spray outlet 16 controlled by a screw 18. The syringe chamber is filled with a DNA vector consisting of a plasmid containing a 1.8kb fragment of the ovalbumen gene and the neo gene together with 0.1 to 1.0μm particles of tungsten as a carrier.

Embryos were incubated for two days and the egg opened so as to expose them. The eggs were rotated so that the embryos lay beneath the shell but the germinal crescents of the protected embryos were exposed. The DNA-coated particles were introduced into the cells of the germinal crescent using the syringe as a ballistic device. Histological studies of the germinal crescent showed that some particles of tungsten had penetrated into the primordial germ cells.

Eggs containing embryos transfected with DNA using this technique were resealed and returned to the incubator. Hatchings were raised to sexual maturity and 3 cockerels were milked to obtain semen. Sperm DNA was extracted using phenol/chloroform, cut with Bam Hl, ECo Rl, & Hind III & Southern blots prepared and hybridized to a ^{32}P labelled neo probe. The results (figure 5) show that the foreign DNA is present in the sperm from ballistic treated birds (1, 2 & 3) but absent from control animals (4, 5 & 6).

It is surprising that the technique works.

Because the germinal crescent is covered by overlying egg-white (albumen) of a thickness of 1-2mm, it was unpredictable what would reach and enter the primordial germ cells.

The cockerels which had been ballistically treated as embryos were subsequently bred. Cockerel 1 is bird that was shown to contain the plasmid-inserted DNA in its sperm. This bird was bred with control hens of the same line zero White Leghorn stock and the fertile eggs incubated to produce chicks. Blood from these birds was used to prepared DNA and 5 μg samples were used to prepared dot blots that were probed with ^{32}P labelled neo. The results showed that 5 out of 29 of these hatchlings were positive (Figure 6). Some samples, such as the bird in row 2, clearly contain many copies/cell as judged by standards (bottom row).

The DNA of these positive offspring was analysed further by Southern blotting (Figure 7). Cutting the DNA with Pst I showed 2 fragments at 2.3 and 0.9 kb that hybridized to the neo probe. In the case of one bird (right, Figure 7) two additional bands at 4.2 and 3.2 kb may indicate junction fragments at integrated sites. These bands are in keeping with the map of the plasmid used in the ballistic treatment (Figure 8).

These data confirm that plasmid DNA introduced ballistically into the primordial germ cells of the embryo can be recovered in the DNA of its sperm and transmitted to its offspring via the germ-line.

CLAIMS

1. A method of transforming cells of a bird with exogenous nucleic acid comprising introducing into said cells particles having said nucleic acid adsorbed to the surface thereof.

2. The method according to claim 1 wherein said cells are germinal crescent cells.

3. The method according to claim 1 or 2 wherein said nucleic acid is DNA.

4. A method according to any preceding claim wherein the particles are of diameter 0.1-1.0 μm.

5. A method according to any preceding claim wherein the particles are of metal.

6. The method according to claim 5 wherein said particles are gold or tungsten.

7. A method according to any preceding claim wherein the particles are introduced into the cells by particle bombardment.

8. A method according to claim 7 wherein particle bombardment is effected without subjecting the tissue of the cells to vacuum.

9. A method according to claim 7 or 8 which employs a needle-less injection syringe.

10. A method of transforming avian cells substantially as described herein with reference to and as illustrated in the accompanying drawings.

11. An avian germinal crescent cell containing a microparticle having exogenous nucleic acid adsorbed

to the surface.

12. A bird including the cell according to claim 11.

13. A method of producing a transgenic bird comprising using the method of any of claims 1-10 to introduce foreign nucleic acid into the primordial germ cells of a bird so that transformed primordial germ cells are produced; allowing a bird containing said transformed primordial germ cells to mature; and breeding said matured bird so that an offspring is produced containing said foreign nucleic acid, said offspring being a transgenic bird.

Patents Act 1977
Examiner's report to the Comptroller under Section 17
(The Search report)

-14-

Application number
GB 9419284.6

Relevant Technical Fields

(i) UK Cl (Ed.M) C3H (HB7T, HB7X)

(ii) Int Cl (Ed.5) C12N 5/10, 15/87; A01K 67/027

Search Examiner
MR C SHERRINGTON

Date of completion of Search
12 DECEMBER 1994

Databases (see below)
(i) UK Patent Office collections of GB, EP, WO and US patent specifications.

(ii) ONLINE DATABASES: WPI, CLAIMS, DIALOG/BIOTECH

Documents considered relevant following a search in respect of Claims :-
1 TO 13

Categories of documents

X: Document indicating lack of novelty or of inventive step.

Y: Document indicating lack of inventive step if combined with one or more other documents of the same category.

A: Document indicating technological background and/or state of the art.

P: Document published on or after the declared priority date but before the filing date of the present application.

E: Patent document published on or after, but with priority date earlier than, the filing date of the present application.

&: Member of the same patent family; corresponding document.

Category	Identity of document and relevant passages		Relevant to claim(s)
X	WO 93/05815 A1	(FILLER, AARON GERSHON) whole document	1
X	WO 93/08292 A1	(AGRACEUTUS INC) whole document	1
X	WO 93/24626 A1	(LARIONOV, OLEG ET AL) whole document	1
X	WO 91/00359 A1	(AGRACEUTUS, INC) whole document	1
X	WO 91/07487 A1	(DUKE UNIVERSITY) whole document	1
X	BioTechnology 1992, 10, 286-291 Transformation of microbes, plants and animals by particle bombardment		1

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